CONTRIBUTIONS TO OUR KNOWLEDGE OF SOIL-FERTILITY.

No. xvi. The Search for Toxin-Producers.

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In the earlier papers of this series, it was shown that bacterio-toxic substances were sometimes present in the clear liquid obtained by shaking a soil with water and filtering it through porcelain. The toxins were potent, for, when the same portion of soil was extracted a second time with water, the extract was highly nutritive. The first extract undoubtedly contained substances which functioned as toxins, as well as substances which acted as nutrients, and, of these two, the toxins were the more powerful.

The local soils were found to be toxic during the cold winter months and not in the dry summer. The toxicity was variable, and even in the favourable season, one could not be certain that a soil, undoubtedly toxic at one time, would be toxic at another. It seemed to be a matter of chance, but this was undoubtedly due to the fact that the cause of this toxicity was unknown. With the elucidation of the origin of the toxins, it will be better understood when a soil is likely to be toxic or otherwise.

Soil-toxicity, as exhibited in the extracts of the soils, can be demonstrated directly and indirectly. Directly, by adding a certain number of a test-bacterium, and obtaining a diminution in that number after an incubation-period of, say, 20 hours at 22°. Indirectly, by obtaining an increased growth as a result of boiling the extract and also of diluting it. A toxic soil exhibits all three characters. There are other points connected with toxic soils, and these will be found in my former papers.

During my earlier work, I was led to believe that the cause of the toxicity would be found in the products of the bacteria,

and, in the beginning of this research, into the etiology of toxicity, some of the more likely bacteria were tested with more or less completeness. The work was tentative, the bacteria were tried one way and another. A favourable result was occasionally obtained, which led to repeated trials with slight variations, but these ended in failure to obtain a truly toxic condition. Some moulds were also tested, but the experiments with these did not lead me to believe that the source of the toxins would be found in the flora of the soil. The fauna remained, and when the amœbæ were tested, the first results were so satisfactory that the source of the toxins seemed to have been traced. Unfortunately, these results were not confirmed, and, as the work proceeded, it became evident that the toxic effect was caused by the production of alkali in the solutions in which the protozoa were growing. The test-bacteria were very sensitive to changes of reaction, and, so far as the solutions were concerned, it was made clear that a perfectly neutral solution was exceedingly difficult to maintain, and that any departure from a strict neutrality retarded the growth of the bacteria and exhibited some of the effects of a toxic solution. The main steps of the work that led to this conclusion are recorded in the following pages. Many experiments have been omitted because the results did not appear to justify a lengthening of the paper. They were either indefinite or confirmed a negative result previously obtained.

It is well known that some soil-bacteria are inimical to others, and it was considered that some one group might be specially so to bacteria generally. Instances of an inhibiting or toxic effect exercised by some micro-organisms against others are familiar to most workers in soil-bacteriology. It is an ordinary experience to find certain colonies of bacteria, such as *Bac. mycoides* and certain moulds, passing over or through other bacterial colonies when spreading over the surface of an agar-plate. Occasionally, they are seen to avoid some particular colony, and we find these surrounded by a clear zone of agar, across which the wandering mould or bacterium will not pass. Apparently, the colony has sent into the medium some toxic substance, and there does not

appear to be a thinning away of the toxin, for the wandering colony generally becomes heaped up at the margin of the toxic zone and then spreads round and eventually encircles the colony. It may be that the thickening of the edge is caused by the toxin becoming so weak as to be able to exercise a stimulating action like other weak poisons.* The Actinomyces-colonies are generally toxic to such wandering moulds and bacteria.

As soil contains so much sand and inert matter, it did not appear to be a suitable medium for experiments with these presumable toxin formers, and it was considered that a fluid medium would be better. With regard to the nutrient added to the water, Bottomley's work with auximones had suggested the idea that there might be a relation between them and decayed soil-toxins. Bottomley used moist peat-moss as a culture-material, but, as this could not be obtained, the first experiments were made with washed sphagnum-moss suspended in water.

The moss was picked, dried, cut up, washed, and again dried. Ten grams were put into a flask with 600 c.c. of tap-water and sterilised.

Two bacteria, T.P.2 and T.P.4, were selected as being possible toxin-producers. They were not identified at first, for, in experiments such as these, one determines the value of the bacterium first and identifies it afterwards, if its activity justifies the identification.† The bacteria were seeded into the flasks of sus-

* Journ. Roy. Soc. N. S. Wales, 1916, p.77.

† Bac. T.P.2 is a short, motile rod measuring $0{\cdot}5 : 2\mu$. Gelatine is liquefied slowly, the colonies being round, white, and slightly raised with a ciliate edge. On agar, the growth is ivory-white, rough and cohesive. Long, needle-shaped crystals are quickly formed in agar containing sodium phosphate, and these are fairly characteristic of the organism. In some media, slime is formed from dextrose, although none is produced on Lipman-Brown agar. In bouillon, the growth produces turbidity, a film and a sediment; nitrates are not reduced. Milk is coagulated and slowly peptonised. On potato, the growth is yellow-brown. The indol test is positive, the Gram test negative, and the bacillus produces neither gas or acid from dextrose, saccharose, mannite, or glycerine. Nitrate is not produced from ammonia salts. It appears to have *Bac. inunctus* as its closest ally.

pended moss, which were incubated at 22° for varying periods.

The extract was prepared in the usual way by filtration first through paper, then through porcelain. One portion of the extract was boiled for an hour under an inverted condenser. The various portions, generally 50 c.c., of the raw, boiled, and diluted extracts, were each seeded with 1 c.c. of a suspension of *Bac. prodigiosus*, and incubated at 22° for 20 hours, when counts were made by the plate-method. The numbers of bacteria in the raw extracts were taken as 100, and those in the treated extracts were calculated in terms of this. Fractions were omitted, and numbers less than 1 were taken as 1. The actual number of the water-control can be found by dividing the extract by the extract/water ratio, for the latter was obtained by dividing the extract-count by the water-count.

Experiment i.

	Growth of *Bac. prodigiosus* in extract, 20 hours at 22°.							
Micro-organism.	T.P.4	T.P.2				Penicillium cladosporioides.		
Nature of moss.	new	new	old			new	old	
Duration of test; days—	29	8	12	11	10	13	10	11
Number of test.	1	2	3	4	5	6	7	8
Extract, boiled ...	766	223	72,000	600	5,500	34	184	1,167
Extract, raw	100	100	100	100	100	100	100	100
Extract, raw, 80% ...	33	54	122	92	100	479	90	48
Extract, raw, 60% ...	15	15	133	100	100	2,240	67	40
Extract, raw, 40% ...	6	4	300	107	100	600	56	14
Extract, raw, 20% ...	2	1	167	107	135	21	36	40
Water-control	1	1	111	77	88	1	25	8
Extract/water ratio ...	417	580	0·9	1·3	1·2	111	4	12

In looking over the results of Experiment i., it is seen that moss is not a good substance for determining toxin-production. When new, it is too nutritive, and when old, that is, when it had been used and washed once or twice, it is too poor. The dilution-curves of tests 3, 4, and 5 are almost horizontal lines, indicating that the extract is of a nature similar to water. The results obtained by boiling the extract in tests 3 and 5 cannot be ex-

plained; they certainly indicate a degree of toxicity which is not confirmed by the dilution-numbers.

The numbers with *Penicillium cladosporioides* are peculiar. A flask with new moss had been sterilised and allowed to stand for several weeks before being infected with Bac. T.P.2. At the end of the incubation-period, the mould was plainly seen growing as a floating mass upon the surface; it had ousted the bacteria, for few bacterial cells were obtained from the fluid. The rise in the numbers upon dilution is typical of a toxin, but the reduction upon boiling is not. Again, the dilution-effect was not obtained in tests 7 and 8.

On the whole the experiment was unsatisfactory, and consequently other media were tried.

Experiments were made with dilute solutions of nitrogenous salts such as ammonium sulphate, ammonium phosphate, and potassium nitrate, as well as with alkaline salts as potassium phosphate, but there was little sign of any probable formation of toxin with any of them.

The use of Collodion-Capsules.

In an endeavour to improve the experimental method, use was made of collodion-capsules, as it was considered that by growing the bacteria outside and the test-organism inside the capsule, the production of toxin might be rapidly determined. Accordingly, capsules were prepared by coating the insides of 3/4 in. test-tubes with 4% agar in water. After drying at 37°, they received one or two coats of thick collodion, and a narrow paper-scale was fixed inside near the middle, and a short length of thin tubing with a thread attachment near the top. The separation of the capsule from the tube was effected by filling the tube with water and slowly raising it to near the boiling-point, when the collodion separated easily from the glass. The capsules were washed in changes of water, steamed to get rid of all traces of alcohol, and finally inserted in wide test-tubes, which were plugged. The medium was added, 20 c.c. in the outer tube, and 5 c.c. in the capsule, and the whole was sterilised. The outer liquid was

seeded with soil-organisms and incubated for some days, when a suspension of the test-organism, *Bac. prodigiosus*, was added to the capsule. After incubation at 22° for a day or two, the cells were distributed, either by blowing air through the culture or by repeatedly drawing up and blowing out the liquid in a pipette. Then 1 c.c. was abstracted, and a count of the bacteria made. Previous to the mixing, the height of the liquid on the paper-scale was read, and afterwards the volume was determined by water run from a burette to the same mark.

The soil-organisms were obtained from a garden-soil that had been kept some time in the laboratory. The numbers of bacteria, originally high, as the soil had been heated, had fallen to that normal for the soil, viz., to 6 to 8 millions per gram. Plates of Lipman-Brown agar were smeared with the soil-suspension in appropriate dilution, and, after several days, it was noted that the flora consisted roughly of 30% of bacteria, and 70% of Actinomyces-forms. Of the latter, 17% were *Actino. chromogena*, which darkened nutrient agar strongly; and 23% were *Actino. odorifera*, which darkened the agar slightly; the remaining 30% were indefinite. Many colonies were picked from the plates, the micro-organisms were classified or grouped, and representatives of the groups used for infecting the liquid outside the collodion-capsules in the tubes.

The experimental results were noted as the progeny of one test bacterial cell originally added to the collodion-capsule, but as nothing will be gained by giving the exact numbers, the general indications, as compared with control-tests obtained from the figures in the various experiments, are here noted.

Experiment ii. Soil-extract [soil 1, water 2 parts].

Soil-bacteria, 6 days at 18°; test-bacteria, 1 and 7 days at 22°.
Inhibiting strongly—Rhizobium from soil, Bac.A7.
Inhibiting slightly—Various (5) Actinomyces-forms, Bac. A5.
Accelerating slightly—*Aspergillus* sp., Bac. A2.
Accelerating strongly—Bac. A1.

As an indication of the value of the terms, it may be said

that the control-test showed that one cell had, in 7 days, increased to 118,000; the Rhizobium had multiplied only to 100; the Actino.-forms averaged 53,000, Aspergillus 240,000, and Bac. A1 had a progeny of 7·28 millions.

Experiment iii. Gum-acacia, 0·2%.

Soil-bacteria, 12 days at 18°; test-bacteria, 5 days at 22°.

Inhibiting slightly—*Aspergillus* sp., *Penicillium* sp., *Actino. chrom.*

Indifferent—*Actino. odor.*, Bac. A1, Bac. A5.

Accelerating strongly—Bac. A17.

Experiment iv. Hay-infusion, 0·05%.

Soil-bacteria, 11 days at 16°; test-bacteria, 1 day at 16°.

Inhibiting—Bac. B4.

Inhibiting slightly—Bac. B5.

Indifferent—Bac. B2, grey and white Actinomycetes.

Accelerating—Bac. B8.

Experiment v. Hay-infusion, 0·05%.

Soil-bacteria, 11 days at 16°; test-bacteria, 2 days at 15°.

Inhibiting strongly—Rhizobium (from soil).

Inhibiting moderately—Bac. A1, Bac. A2.

Inhibiting slightly—Various Actinomycetes, Bac. A5, Bac. A7, Bac. A9.

Experiment vi. Albumen, 0·1% in tube, water in capsule (albumen coagulated upon steaming).

Soil-bacteria, 7 days at 15°; test-bacteria, 1 day at 22°.

Inhibiting strongly—Rhizobium (from soil), Bac. A7.

Inhibiting slightly—Bac. T.P.2, Bac. B4, Bac. C2, Bac.C3, Bac.C1.

Indifferent—Bac. A17.

Experiment vii. Soil-extract [soil 1, water 1 part].

Soil-bacteria, 11 days at 15°; test-bacteria, 1 and 3 days at 22°.

Inhibiting strongly—Rhizobium (from the Lupin), Bac. A7.

Inhibiting moderately—Bac. C3.

Inhibiting slightly—Bac. T.B.2, Bac. B8.

Indifferent—Bac. A1, Bac.A17, Bac.C2.

Experiment viii. Hay-infusion, 0·1%, with dextrose 0·1%.
Soil-bacteria, 12 days at 16°; test-bacteria, 2 days at 22°.
Inhibiting moderately—*Penic. cladosporioides.*
Inhibiting slightly—Actino. 11, Bac. A8, Bac. T.P.3, T.P.4, T.P.5, T.P.6, T.P.7, S.B.2, S.B.5.
Indifferent—Actino. 10, Bac. S.B.1, S.B.4.

The results of the experiments, as a whole, showed that the action of the soil-microbe is generally irregular. This is notably the case with Bac. A17, which is the same as Bac. A1. In some cases, it increased the growth of the test-organism, while, in others, it had an indifferent action. In the case of Rhizobium and of Bac. A7, we have bacteria which produce a luxuriant slime in media containing sugar, and even in those containing merely a trace of sugar or none at all, the inhibiting property is seen to remain. I cannot but think that they acted by extracting the nutrient from within the capsule in order to produce their slime, which remained in the outer tube. The inhibition is, therefore, in all probability, due to the absence of nutrients rather than to the presence of toxins in the collodion-capsule. As the experiments did not promise to aid the investigation, they were discontinued.

The Use of various Media.

The action of Rhizobium led to its being tested in flasks of various media, and the tests are grouped together in the following Table.

EXPERIMENT IX.

Soil-organism ...	Rhizobium.			
Derivation	Soil.	Soil.	Lupin-nodule.	Lupin-nodule.
Medium	Hay-infusion, 0·1%.	Gum-acacia, 0·2%.	Dried blood, 0·4%.	Ammonium sulphate, 0·1% with salts
Duration of test ...	3 days.	16 days.	6 days.	11 days.
Temp. of incubation	16°	15°	15°	16°
Number of test ...	1	2	3	4
Extract, boiled ...	1,684	343	325	41
Extract, raw... ...	100	100	100	100
Extract, raw, 80% ...	170	99	106	52
Extract, raw, 60% ...	106	69	96	15
Extract, raw, 40% ...	80	63	67	5
Extract, raw, 20% ...	175	44	49	18
Water-control ...	58	23	15	22
Extract/water ratio...	1·7	4·3	6·5	4·6

There are indications of the formation of toxin in some of these tests, although it must be said that they are not very pronounced. Test 1 shows a narrow extract/water ratio, pointing either to the possible absence of nutrients in the extract or to the presence of toxins. The increases obtained in the boiled and in the diluted extract lead one to believe that toxins were present. Test 2 had undoubtedly been incubated for too long, but there is a suspicion that the extract is of the same nature as test 1. Test No.4 is irregular, and, so far as we can judge, gives us no information.

In these tests, the growing organism had undoubtedly removed nutrients from solution, and, by utilising them, had prevented their appearance in the porcelain-filtered extract. This was shown by an extension of test 1, in which the raw, uninoculated, filtered hay-infusion gave 666,800 cells, and the same, when boiled, gave 379,600 cells as against 100 of the unboiled bacterial extract.

A mixed culture of bacteria, existing as an actively nitrifying suspension of soil-bacteria, was seeded into a solution of 0·1% dextrose in 0·1% hay-infusion. The suspension was incubated at 15° for 7 days. On preparing and testing the extract, it was

found to give the boiling- and dilution-reactions. The original mixture of bacteria was plated out in levulose ammonium-sulphate agar, and the flora was seen to consist of five organisms named provisionally S.B.1-5. These were tested with the same hay- and dextrose solution, but as they showed no sign of toxin-formation, it is unnecessary to give the details. The original experiment was repeated, but the result was quite different from the first.

EXPERIMENT X.

Soil-organisms	Mixed bacteria.	
Medium	Hay-infusion, 0·1% with dextrose 0·1%.	
Duration of test	7 days.	12 days at 15°.
Extract, boiled	917	96
Extract, raw	100	100
Extract, raw, 80%	176	92
Extract, raw, 60%	230	59
Extract, raw, 40%	615	31
Extract, raw, 20%	465	7
Water-control	28	1
Extract/water ratio	3·5	472

The differences between these tests is marked, and, while there probably was a difference in the kinds of bacteria added in the two cases, there is the suggestion that the time of incubation may have an influence upon the result.* In the 12-days' test, the toxin may have been formed on or about the 7th day, and have

* The experiment was repeated some months afterwards, with the following results:—

Soil-organisms.	Mixed bacteria.	
Duration of test.	2	7 days.
Extract, boiled	188	165
Extract, raw	100	100
Extract, raw, 80%	32	48
Extract, raw, 60%	8	40
Extract, raw, 40%	1	34
Extract, raw, 20%	1	26
Water-control	1	9
Extract/water ratio...	100	1·2

The previous experiment was not confirmed, and there is no evidence of toxicity shown by the dilution-curves.

become decayed by the 12th It appeared advisable, therefore, to test the solutions, from time to time, to see the influence of the time-factor. This was done in the following experiment, in which Bac. T.P.2 was used at 16° to 18°.

EXPERIMENT XI.

Soil-organism	Bac. T.P.2.						
Medium	Hay-infusion, 0·1%.				Hay-infusion and dextrose, 0·1%.		
Duration of test in days	3	5	7*	11	3	5	11*
Extract, boiled... ...	450	308	70	35	240	560	2,092
Ratio of boiled extracts	12·8	8·8	2	1	1	2·3	8·7
Extract, raw	100	100	100	100	100	100	100
Extract, raw, 80% ...	90	80	122	99	55	36	84
Extract, raw, 60% ...	48	107	118	76	32	24	107
Extract, raw, 40% ...	31	111	159	63	17	19	123
Extract, raw, 20% ...	16	82	111	58	8	7	73
Water-control	5	56	122	32	1	9	192
Extract/water ratio ...	18	1·8	0·8	3·1	113	11·5	0·5

* The numbers in these are irregular, largely owing to the counts being low.

During the filtration of the infusions through the porcelain, it was noted that, in the dextrose-media, a considerable amount of slime had been formed. Attention was not called to this in the three days' culture, but, in the five and eleven days' culture, the slime was pronounced. It is clear from the results that the organism had produced a certain amount of toxin about the sixth day in the simple hay-infusion, and that it had largely disappeared by the eleventh day. The ratio of growth, which is a rough index of the approximate amounts of nutrients in the extract, declined as time went on, and so did the effect of boiling the solution. In the hay- and dextrose-solutions, boiling showed a steady increase of nutrient produced as the age of the culture increased, while, without dextrose, the reverse was the case.

The organism T.P.2 was originally obtained upon an agar-plate which had been seeded with a suspension of soil-bacteria. It was conspicuous in producing a zone across which neither moulds nor spreading bacteria would go. The single colony upon purification was found to consist of two closely allied forms pro-

visionally named T.P.1 and T.P.2, the former producing a white colony, the latter an ivory-white on nutrient agar. T.P.1 liquefies gelatine quickly and forms no slime on dextrose-media, while Bac. T.P.2 liquefies gelatine slowly, and forms a slime from dextrose. The belief was raised by other experiments that the formation of slime withdraws nutrients from the medium, and yields a poor extract; but whether the toxin-formation has anything to do with the production of slime, has yet to be determined. Bac. T.P.1, the slime-free ally of Bac. T.P.2, was tested under the conditions of the last experiment, viz., in hay-infusion, with and without dextrose, and at periods of 3, 6, and 10 days, but no evidence of toxin-formation was obtained.

An attempt was made to determine the effect of slime-formation upon the production of toxin by using a race of Rhizobium from the soil, which formed a luxuriant slime on solid dextrose-media.

Experiment xii.

Medium.	Hay-infusion, 0·1%.			Hay-infusion with dextrose, 0·1%.	
Soil-organism.	Rhizobium.			Rhizobium.	
Duration of test in days.	3	6	13	3	6
Extract, boiled	82	5	205	20,840	45,030
Extract, raw	100	100	100	100	100
Extract, raw, 80% ...	13	10	84	3	40
Extract, raw, 60% ...	1	5	77	2	36
Extract, raw, 40% ...	1	4	58	2	49
Extract, raw, 20% ...	1	3	50	2	47
Water-control	1	2	35	1	37
Extract/water ratio ...	1,900	49	2·8	72	3

A 13-days' test with hay-infusion and dextrose was found to be sterile, and has been omitted. Although these tests were intended to see the effect of slime-production upon toxin-formation, it happened that no slime was obtained on the porcelain filter from the dextrose solution, and, but for the growths on agar-slopes, one would have thought that the cultures were dead. However, the slow reduction of the extract/water ratio as time went on, and the great increase obtained on boiling the dextrose-solutions were again noted.

I have found, in the past, that soils are more toxic in the winter than in the summer-months, and it is to be expected that temperature will have some influence in either the production or the decay of the toxin. The influence of temperature was tested in the following.

EXPERIMENT XIII.

Medium	Hay-infusion, 0·1%.				
Soil-organism	Bac. T.P.2.				
Temperature	15·5°.			22°.	
Duration of test in days ...	2	4	8	2	7
Extract, boiled	20	74	123	30	16
Extract, raw	100	100	100	100	100
Extract, raw, 80% ...	33	77	48	46	29
Extract, raw, 60% ...	12	64	19	26	12
Extract, raw, 40% ...	3	45	12	17	7
Extract, raw, 20% ...	1	45	10	13	5
Water-control	1	53	11	6	5
Extract/water ratio ...	248	2	9	15	18

The experiment was rather disappointing, as there was little evidence of toxin-formation at the lower or the higher temperature. In four days at 15·5° it is shown by the low extract/water ratio that much of the nutriment has been locked up in the bodies of the bacteria. As the ratio is higher on the eighth day, one would imagine that there had been some dissolution of the cells.

In an earlier experiment, there had been signs that some formation of toxin had occurred in a solution of gum-arabic infected with Bac. T.P.2. This led to the employment of an old culture of Rhizobium which had been grown in hay-infusion and dextrose, and which contained a quantity of slime. It was sterilised and infected with Bac. T.P.2. It showed no signs of toxicity on the third day.

An old culture of Bac. T.P.2 in hay-infusion was sterilised and infected with Bac. T.P.2, but there were no signs of toxicity on the second or fourth day.

A solution of Gum-Acacia, 0·2%, when infected with Bac. T.P.2, showed no toxicity on the second or fourth day.

A rod-shaped bacillus, Bac. A17, which had appeared to stimulate the growth of *Bac. prodigiosus* in collodion-capsules, was tested in hay-infusion, but there was no evidence of toxin formation in the boiled or diluted extracts.

In view of the indeterminate nature of the results hitherto obtained, it was deemed advisable to augment the intensity of the growth of the bacteria by increasing the amounts of the nutrients and by aërating the media during cultivation. To gain this end, beakers containing cotton-wool were sterilised, and media, such as 1% hay-infusion with and without 1% dextrose, which had been seeded with various organisms, were added in sufficient quantity to moisten the wool. After incubation at 22°, the cotton-wool was squeezed and washed, and the liquids made up to a definite volume before being filtered through porcelain.

A soil-Rhizobium, Bac. T.P.2, *Penicillium cladosporioides*, and an Actinomyces, A10, were tested at different times, such as 3, 6, and 11 days; but, in none of the tests, was any evidence of toxin-formation demonstrated. This also applied to old cultures of bacteria reinfected with moulds. The method was useless, therefore, for the object in view.

The experiments, however, raised the idea that small changes in the reaction might be responsible for much of the irregularity in the results. Bacterial cultures, in the absence of a fermentable sugar, are generally more or less alkaline, while soil-extracts are supposed to be more or less acid. The soils used in this set of researches were acid, that is to say, when a piece of litmus-paper was inserted or pressed into a paste or porridge made by mixing the soil with water, it became reddened in the course of a few minutes. The aqueous extract of such soils should, therefore, be acid, and it appeared that, if the extract could be prevented from becoming alkaline through the action of bacteria, there would be a better chance of demonstrating any development of toxicity. At any rate, the cultural fluids would be more in harmony with the conditions that pertain in the soil. To effect this faint, permanent acidity, it would be necessary to add some substance which would absorb any alkalinity produced, in the

same manner that chalk maintains a faint acidity in fermenting solutions of sugar. The only substance that promised to achieve this purpose was humic acid, and its use appeared advisable, as it is ever present in soils.

A quantity of humic acid was accordingly prepared from rotted bamboo-mould by treatment with sodium hydrate, followed by acidification, washing with water by decantation, filtration, and finally by dialysis. The acids were dissolved in normal soda and sterilised.

Tests were made with strong hay-infusion with and without dextrose by the cotton-wool method, but, although several organisms were used, no results of any importance were obtained: the solutions were far too nutritive. A converse test with tap-water and no nutrient showed that the humic acid acted as a poison. Although an opalescent suspension of bacteria had been added, no living bacteria were to be found by the third day. In one case, a pink yeast had obtained access to the flask, and, on the thirteenth day, the extract showed a rise upon boiling; and, upon dilution with dilute hay-infusion, the same sterility, towards the introduced bacteria, was noted after a few days. The humic acid was undoubtedly the cause of the phenomenon. It had been dissolved in normal soda, and the solution was added to the flask of medium, and was followed by the quantity of normal hydrochloric acid necessary to neutralise the soda. The humic acid remained largely dissolved, and acted as a soluble acid. In one test, that of the pink yeast on the thirteenth day, the acidity of the extract to phenolphthalein was $= +0{\cdot}5^{c}$ by Fuller's scale, that is the extract contained 0·5 c.c. of normal acid per litre. The experiments suggest that, to be effective, the humic acid should be insoluble in water.

Experiments with Humus.

As the toxin-formation in soil must be related to the changes in the organic matter, an attempt was made to see in how far humus would be useful in elucidating the problem. Rotted bamboo-grass was sifted, partly dried, mixed and sifted again to obtain a uniform mould. Portions weighing 10 grams were put into deep Petri-dishes, and sterilised by heating for two hours at

130°. When cold, 10 c.c. of a suspension of Bac. T.P.2 were added to each portion. After various periods of incubation at 18°, a portion was transferred to a shaking bottle, treated with 500 c.c. of distilled water, and shaken 50 times at 10-minute intervals for an hour. The suspension was then filtered through paper and porcelain, and the usual procedure followed.

Experiment xiv.a.

Soil-organism	Bac. T.P.2.	
Duration of test	3 days.	6 days.
Extract, boiled	3,870	210
Extract, raw	100	100
Extract, raw, 80%	50	79
Extract, raw, 60%	13	50
Extract, raw, 40%	9	21
Extract, raw, 20%	5	4
Water control	1	2
Extract/water ratio... ...	150	52

As the experiment did not seem to be going to give any useful information, the remaining portions were extracted with different quantities of water to test the influence of various strengths of extract.

Experiment xiv.b.

	Growth of *Bac. prodigiosus* in extracts of leaf-mould infected with Bac. T.P.2, and incubated for 11 days at 18°.		
	10-gram portion to water.		
	500 c.c.	300 c.c.	100 c.c.
Extract, raw ...	100	100	100
Extract, raw, 80% ...	35	240	487
Extract, raw, 60% ...	5	276	1,563
Water-control ...	23	3	46
Extract/water ratio ...	4	36	2

The results of Experiment xiv.a, are much the same as have been obtained in solutions, viz., a lessening of the nutritive and of the boiling effects, as the period of incubation proceeded. In Experiment xiv.b, we have the dilution-effect becoming more pronounced as the water used in making the extract became less.

Portions of the air-dried mould containing 14 grams of dry matter were put into Petri-dishes and sterilised. They were infected with cultures of Bac. T.P.2, and of Actinomyces 10, and the moisture raised to 40%. The extracts were prepared by shaking each portion with 500 c.c. of distilled water.

EXPERIMENT XV.

Soil-organism ...	Bac. T.P.2.				Actino. 10.		Control.
Duration of test in days	4	6	18	24†	10*	18†	24†
Extract, boiled ...	44	164	119	47	71	90	138
Extract, raw ...	100	100	109	100	100	100	100
Extract, raw, 80% ...	849	532	270	66	292	171	119
Extract, raw, 60% ...	3,710	1,880	1,845	54	574	268	68
Extract, raw, 40% ...	9,500	3,090	6,637	37	1,038	160	57
Extract, raw, 20% ...	8,070	2,500	16,090	17	562	5	25
Water-control ...	47	20	153	1	2	1	1
Extract/water ratio ...	2	5	0·6	1,130	44	275	460
Reaction	+0·6	+0·6	+0·55	+0·3	—	+0·4	+0·2

* Growth of a green Penicillium at edge of dish.
† Permeated with Penicillium.

The growth of the accidentally introduced Penicillium resulted in a flattening of the dilution-curves, and in a diminution of the acidity. From this, it must be inferred that the acidity was chiefly responsible for the toxicity of the extracts. To test the matter, a further experiment was made with partially neutralised mould, and with neutralised extracts.

Four-ounce glass bottles were used instead of Petri-dishes, and, into each, 18·2 grams of mould, containing 14 grams of dry matter, were introduced. They were sterilised at 130° for two hours, moistened with 3 c.c. of sterile water, and steamed for an hour. The steaming proved to be unnecessary, as portions of the heated soil proved to be sterile. One of the portions was infected with a Fusarium, another with a Rhizobium, a third served as a control, a fourth was treated with enough lime-water to neutralise the apparent acidity, and the last was subjected to the vapour of ammonia for a couple of hours. The moisture in them all was brought to 40%. The extract of the raw mould was found to be very acid, 1000 c.c. containing the equivalent of

one c.c. of normal acid, using phenolphthalein as an indicator. The extracts were neutralised with sodium bicarbonate. The Fusarium, the Rhizobium, and the control-tests were incubated for five days at 22°.

Experiment xvi.

Soil-organism ...	Fusarium sp.		Rhizobium (soil).		None.			
	acid.	neutral.	acid.	neutral.	acid.	neutral.	Lime.	Ammonia.
Reaction of extract ...								
Extract, raw	100	1,074,000	100	8,151	100	114,000	609	29,070
Extract, raw, 80% ...	176	315,400	127	3,200	116	109,000	1,783	43,250
Extract, raw, 20% ...	27,940	218	153,800	38	21,860	791	81,390	46,740
Water-control ...	1,000	—	763	—	1,040	—	—	—
Extract/water ratio ...	0·10	—	0·13	—	0·09	—	—	—
Reaction	+1·0	—	+0·9	—	+1·0	—	+0·75	+0·7

The point to be noted from this set of tests is, that the neutralisation of the acidity of the extract has converted the toxic

into a nutritive condition, not only in the raw extract, but also in the 80% dilution. The numbers obtained in the extracts of the control and of the infected leaf-moulds are of the same order, and indicate that the toxic property is inherent in the sterilised medium. The suspicion is strengthened, that the toxins are really acids developed during the sterilisation of the organic matter. It is evident that the treatment of the leaf-mould with lime-water or with ammonia was not sufficient to neutralise the excessive acidity of the humus, for the numbers show a position intermediate between the acid and the fully neutralised extracts.

In a new set of tests, the sifted mould was treated with water to eliminate the sand with which it was mixed. It was then dried, and again sifted through a finer sieve to remove the bulk of the fine, light fibres. It contained moisture 10·2%, and ash 43·8%, leaving 46% for the organic matter. Portions containing 10 grams of organic matter were put into 4-ounce bottles, and sterilised at 130° for two hours. During the sterilisation, an odour of burnt sugar was noticeable.

A portion of the sterilised leaf-mould was tested against a portion of the unsterilised for acidity. Each was shaken 300 times with 500 c.c. of water, and filtered. This was repeated a third time. The acidity of the filtrates was tested with N/100 soda, using phenolphthalein as an indicator. The figures represent the number of c.c. required to neutralise the 500 c.c. of the extract.

	mould, heated.	mould, not heated.	difference.
1st 500 c.c. ...	47·5	7·5	40·0
2nd 500 c.c. ...	16·25	5·0	11·25
3rd 500 c.c. ...	10·0	3·75	6·25

The curves of these numbers were plotted, and it was seen that the curve of the heated mould would meet that of the unheated mould at the fifth 500 c.c., and that, at the fourth, there would

be a difference of 3 c.c. The total differences would, therefore, amount to 60·5 c.c. of N/100 acid for the total acidity developed during sterilisation and *removable by washing with water*. In terms of the organic matter of the mould, this means that 100 grams during sterilisation developed an acidity equal to 6·05 c c. of normal acid, equivalent to 0·36% grams of acetic acid.

A portion of the sterilised leaf-mould, containing 10 grams of organic matter, upon being gradually moistened with water, was found to form a fairly coherent ball when pressed in the hand after the addition of 15 c.c. of water to each portion. Accordingly, 15 c.c. of water containing 6 c.c. of N/10 sodium bicarbonate were added to each portion, which was thoroughly mixed and pressed down. Suspensions of the bacteria in 1 c.c. of water were subsequently added, mixed, and pressed.

After an incubation of five days, extracts were made, and their effect upon the growth of *Bac. prodigiosus* noted.

Experiment xvii.

Soil-organism... ...	Bac. T.P.2.		Rhizobium.		None.	
Reaction of extract ...	acid.	neutral.	acid.	neutral.	acid.	neutral.
Extract, boiled ...	106	—	60	—	152	—
Extract, raw	100	77,930	100	41,960	100	85,800
Extract, raw, 80% ...	132	70,580	281	35,420	108	89,310
Extract, raw, 20% ...	1,677	4,390	5,342	12,740	1,000	9,950
Water-control ...	1,471	—	200	—	1,316	—
Extract/water ratio ...	0·07	—	0·5	—	0·07	—

The numbers in the control and in the T.P.2 tests are virtually the same, and evidently no growth of the bacillus had occurred. The numbers with Rhizobium are different, not only in the acid extract, but also after it had been neutralised with soda. The fact that Rhizobium can grow in the partially acid leaf-mould is a point worth noting. It is needless to discuss the possible formation of toxins with these leaf-moulds, and the re-

maining portions of the tests were discarded until further work had been done.

The addition of the bicarbonate of soda to the portions of leaf-mould did not neutralise the soluble acidity, for when the raw extracts were tested, it was found that the T.P.2 test had an acidity equal to +0 62° in the extract. The control had +0·63°, and Rhizobium +0·35°. It is, perhaps, to be expected that, in a substance like rotted leaf-mould, there will be a mixture of humic acids, some soluble in water, some partially soluble, and some insoluble in water. The humates are so complex that it was a mistake to consider that even all the water-soluble acid would have been obtained by water-extraction, unless the time-factor had been taken into account, and days instead of hours been occupied in the extraction.

The necessity for having a neutral vegetable-mould was emphasised, especially in the last two tests, and some experiments were made with the object of getting a better idea of the true acidity.

The Acidity of heated Leaf-mould.

A number of portions of the leaf-mould, each weighing four grams, were bottled, and some were heated for two hours at 130°. A sterile and an untreated portion were repeatedly shaken up with a standard alkaline solution at intervals during the time of contact, and filtered. The filtrates were tested for residual alkalinity or, when neutral liquids were used, for acidity. The numbers represent cubic centimetres of normal acid in, or derived from, 100 grams of the dry, organic matter of leaf-mould.

Calcium bicarbonate.—The acidity was first tested by the method of Hutchinson and MacLennan,* which consists in having the portions in contact with a solution of bicarbonate of calcium for a certain time, and subsequently determining the amount of lime that had been removed from solution

Heated	...	...	...	93·3
Control	...	...	...	91·7

* Journ. Agric. Sci., vii., 75.

Sodium bicarbonate.—Portions were moistened with 10 c.c. of alcohol, and treated with 100 c.c. of water containing 1 gram of bicarbonate of soda, for five days.

Heated	165
Control	121

Other portions were moistened with 5 c.c. of alcohol, and treated with 200 c.c. of water containing 0·8 grams of bicarbonate.

Heated	82·4
Control	75·5

Alcohol.—Portions were shaken up with 200 c.c. of neutral spirit and allowed to stand overnight.

Heated...	9·4
Control	4·0

Water.—Portions were shaken up with 500 c.c. of water and allowed to stand overnight.

Heated	24·1
Control	9·2

Lime-water.—Portions were treated with 200 c.c. of N/21 lime-water and allowed to stand overnight.

Heated	383
Control	362

The numbers obtained with lime-water were so high, that the experiment was repeated. The four grams of leaf-mould were shaken with 300 c.c. of approximately N/24 lime-water, and due allowance was made for the amounts removed in the portions of fluid abstracted.

Normal alkali, in c.c., absorbed by 100 grams of dry organic matter.

Days.	1/8	1	2	3	4	5	7	9
Heated	340	403	423	432	442	448	456	462
Control	355	395	410	418	426	432	440	444

This confirms the previous test in showing that a comparatively large amount of lime is removed from solution, and that

more is absorbed by the sterilised than by the unsterilised mould. The organic matter has absorbed from 12 to 13% lime [CaO], and about 90% of the total was absorbed within two days.

The difference in the amount of base absorbed from the hydrate, as against the bicarbonate, led to a test being made with bicarbonate of magnesia. A quantity of freshly precipitated and washed carbonate was suspended in water, and a current of carbon dioxide was passed through for several hours. The solution was filtered, and 200 c.c were added to 4 grams of soil in stoppered bottles. The solution of bicarbonate of magnesia was approximately twelfth normal The bottles were shaken frequently, and the portions abstracted from day to day and boiled with an excess of N/20 sulphuric acid for ten minutes, and titrated with N/20 soda in presence of phenolphthalein.

Magnesium bicarbonate·—Normal alkali, in c.c., absorbed by 100 grams of dry organic matter.

Days.	3	5	7	10
Heated... ...	99	99	105	104
Control... ...	89	89	99	99

The numbers are closely akin to those obtained with calcium bicarbonate, and much under the tests with lime-water.

Baryta-water.—The action of lime-water was controlled by a test made with baryta-water, in which 300 c.c. of approximately N/15 alkali were added to each 4-gram-portion of mould.

Normal alkali, in c.c., absorbed by 100 grams of dry organic matter.

Days.	1	2	3	5	6	7	8	9	12
Heated... ...	389	408	419	434	442	445	448	414	464
Control	365	382	391	400	405	417	418	425	433

These numbers run closely with those of the lime-water test

The set of experiments show that vegetable-mould, originally of an acid reaction, is made more acid by the action of heat, such as by sterilisation for two hours at 130°. The acid substances are partly soluble in alcohol and in water. They consist of two kinds, one of which is capable of being neutralised by the bicarbonates of the alkaline earths. The other makes itself evident when in contact with the hydrates of the earths. The mould which was examined removed from four to five times more alkali from the hydrate than from the bicarbonate.

At the close of the baryta-water experiment, the control-soil was rapidly filtered on the pump, washed with a small quantity of water, transferred back to the bottle and shaken with 300 c.c. of water, and tested from time to time.

3 hours	26·5
1 day	36
2 days	36

Of the 433 c.c. removed from 100 grams of dry, organic matter, 36 were given up to water, and we may, therefore, consider the difference of roughly 400 c.c. as being in combination.

The sterilised leaf-mould was also filtered and shaken with 200 c.c. of twentieth-normal hydrochloric acid. This removed the following amounts of normal lime.

3 hours	328
1 day	343
2 days...	343

Deducting this from the total baryta absorbed, viz., 464 c.c, we have 120 c.c., which is close to the amount absorbed from the bicarbonates. It is evident that the kind of humic acid, which fixes the bulk of the lime from a solution of the hydrate, forms with it a feeble combination. This is destroyed by mineral acids but is not affected to any extent by water. The combination seems to be too definite in its quantitative relations for a simple case of adsorption.

From the experiments, it was concluded that the organic matter of the leaf-mould contained humic acids, and, of these, about one-quarter were capable of decomposing the bicarbonates of the earths, while three-fourths were too weak to do this but

could combine with the earthy hydrates. Heating the mould increased the amount of acid, and the increase was largely soluble in water.

Humus with Calcium Carbonate.

According to the earlier lime-water test, in which 100 grams of dry organic matter absorbed 383 c.c. of N/1 alkali from lime-water, it appeared that a neutralisation of the acids of the leaf-mould would be obtained by adding 2 grams of calcium carbonate to each portion containing 10 grams of dry organic matter, and that neutralisation would be certain if 2·5 grams were added. A set of bottles were prepared, each receiving the equivalent of 10 grams of dry organic matter, and 2·5 grams of calcium carbonate as precipitated chalk. The bottles were sterilised for two hours at 130°, then moistened and stirred with 15 c.c. of water, and steamed for an hour. Subsequent tests showed that sterilisation had been complete, and that the extracts furnished by the tests were neutral to phenolphthalein. Sets were infected with certain micro-organisms and incubated at laboratory temperatures for varying times. It will be remembered that the organisms T.P.2 and Actino. 14 were chosen because they inhibited the growth of spreading bacteria and moulds on agar-plates. A test made at the beginning of this experiment showed that T.P.2 had lost its toxic power, while Actino. 14 had not. In view of this, the bottles of T.P.2, which had been infected, were incubated in an atmosphere of carbon dioxide, to see if this would have any influence in restoring the toxicity. Several of the portions, which were tested on the eighteenth day, were unfortunately destroyed.

EXPERIMENT XVIII.

Soil-organisms ...	T.P.2 (in CO_2).			Rhizobium.		Soil-suspension.		Actino. 14.			Pink yeast.		None (control-soil).	*Penicillium cladosporioides.*		
Incubation in days ...	4	11	26	4	11	7	21	7	14	53	8	26	21	7	14	26
Extract, boiled ...	45	62	25	42	61	7	16	42	28	12	60	22	20	47	36	15
Extract, raw	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Extract, raw, 80% ...	76	91	68	81	87	82	58	108	84	81	85	78	55	102	79	101
Extract, raw, 20% ...	76	75	31	38	54	9	15	17	33	22	61	22	33	60	16	8
Water-control ...	0·7	0·5	0·3	0·3	0·3	0·5	1·4	0·4	0·4	0·2	0·4	0·3	0·3	0·2	0·06	0·04
Extract/water ratio ...	142	205	342	322	322	197	73	225	232	586	260	360	293	477	1,580	2,200

A general glance over the results leads to the belief that no toxins are formed by the organisms. It may be that the excess of carbonate of lime has brought about a condition in which the preservation of the toxins is not possible. The outstanding feature of the experiment is the increased nutritive effect obtained with *Penicillium cladosporioides*. The extract/water ratio increased very rapidly, indicating that the mould had been actively attacking the organic matter and producing substances which stimulated the test-organism, *Bac. prodigiosus*, to an increased production.

The amount of unacted-upon carbonate in the treated leaf-mould led to the belief that too much had been added, and that an excess of carbonate was not desirable.

Humus neutralised with Lime-water.

A quantity of sifted leaf-mould was soaked in lime-water for an hour, 5 litres of lime-water being used for every 500 grams of leaf-mould. A trial test showed that this proportion furnished a neutral extract, although, when the bulk was prepared, the extract of the mould was faintly acid. This was possibly caused by the subsequent sterilisation. At the end of an hour, the suspension was stirred and decanted on to a filter, and the organic matter washed, and at the same time separated from the heavier sand by repeating the procedure with water. The residual organic matter was dried, sifted, and bottled.

At this stage, the mould contained moisture 40·42%, ash 18·80%, and organic matter 40·48%, so that 24·5 grams contained 10 grams of organic matter. This quantity was put up into a number of four-ounce bottles, which were heated for two hours at 130°. Most of the moisture was driven off by the heating. A test showed that the heated mould formed a coherent mass, upon being pressed in the hand, when a weighed portion contained 20 grams of water, and this was accepted as being the quantity requisite for a full bacterial growth. After the heating, the water was raised to 19 c.c., the remaining 1 c.c. being left for the infecting suspension. The water was thoroughly incorporated, and the bottles were steamed for an hour. Sets were infected with several micro-organisms and kept at room-temperature, 15°.

EXPERIMENT XIX.

Soil-organisms ...	*Penic. cladosp.*			Rhizobium.			Actino. 14.		*Fusarium* sp.			Control leaf-mould.	*Pen. clado.* + Rhizobium.		*B. mycoides.*	B.A.17.
Incubation in days ...	10	17	31	10	20	38*	20	38*	20	31	31†	10	10	26*	6	6
Extract, boiled ...	63	128	16	85	42	56	70	51	42	32	7	58	43	41	39	50
Extract, raw ...	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Extract, raw, 80% ...	187	132	32	100	54	77	76	94	118	98	28	58	82	58	50	84
Extract raw, 50% ...	—	—	18	—	—	49	—	64	—	44	27	—	26	35	33	56
Extract, raw, 20% ...	38	51	11	30	13	30	22	18	13	9	3	30	3	21	25	31
Water-control ...	1	1	1	1	1	8	5	6	1	2	1	1	1	7	1	1
Extract/water ratio...	475	440	670	299	585	13	22	16	295	42	204	394	248	14	556	469
Reaction of extract...	+0·1	+0·04	−0·04	+0·18	+0·08	+0·06	+0·02	+0·08	+0·0	+0·0	+0·3	−0·02	+0·0	+0·04	+0·1	+0·1

* Grown at 19° instead of 22°. † Extracted with 250 c.c. of water instead of 500 c.c.

The behaviour of *Pen. cladosporioides* was different in this experiment from that in the presence of an excess of chalk. It did not decompose the organic matter so quickly, but the rise in the dilution-curve was more pronounced. This suggested the idea that, if the nutritive effect could be kept down, the curve might be more accentuated Use was made of Rhizobium for growing with the Penicillium because, when the former was tested at the same time, viz., on the tenth day, the nutritive ratio was lower than either the latter or the control-test. The double growth kept down the ratio, but there was no elevation of the dilution-curve. The growing Penicillium brought about an alteration in the reaction of the extract. The reaction of the control-test seems to indicate, that the bacteria and moulds cause the medium to furnish acid extracts which, in time, may become alkaline.

The effect of using different amounts of water in preparing the extract was shown in the case of the mould infected with Fusarium species. Two tests were extracted at the same time, one with 500 c.c., the other with 250 c.c. of water. The stronger extract gave the higher nutritive ratio, as 204 : 42, while the weaker solution showed a tendency to produce a rise in the dilution-curve.

On the whole, the neutralised leaf-mould did not come up to expectations in serving as a suitable medium for the demonstration of toxin-production by the selected micro-organisms.

When an extract is diluted and subsequently sown with bacteria, it will give a count in proportion to the amount of dilution. But if the extract is acid, the dilution will be less acid, and the count will not be in proportion, but will be more or less according to the effect of the change in the acidity upon the growing bacterium. It is difficult to say just what this difference from the normal would be, especially with such weakly acid extracts as in Expt. xix. The neutralisation of an extract so strongly acid as +1·0 is well seen in Expt. xvi., in which the dilution to one-fifth raised the count from 150 to 300-fold. A set of experiments were made to obtain some definite information regarding the influence of slight variations in the reaction.

The Influence of Reaction upon Bacterial Growth.

An extract of the same vegetable-mould as in Expt. xix. was prepared, and the acidity was determined in the following manner. Twenty-five c.c were pipetted into a beaker, an equal volume of distilled water was added, and the beaker was covered. It was boiled for ten minutes to expel the carbon dioxide, and rapidly cooled. Three drops of phenolphthalein were added, and N/100 soda was run in until a difference in the tint was manifest. An end-point was not aimed at; just the difference in the tint from a yellowish to a faint brown. From the reading, 0·05 c.c. was deducted as being necessary to produce the change of tint over the neutral point. For example, 25 c.c. of an extract gave a tint with 0·15 c.c. of N/100 soda. Deducting 0·05 c.c., the reading becomes 0·1 c.c. for the neutral point. With 0·4 c.c., the tint was a pronounced red, and the final deep purple was obtained with 0·9 c.c. The neutral quantity 0·1 c.c., when calculated to normal acid per litre, gives 0·04 c.c., which, by Fuller's scale, is represented by +0·04. A good, white light is required during the titration to observe the change of tint. The method appears to be correct, for when the extracts were neutralised by the findings of the method, they always gave the highest counts after incubation with the test-bacterium.

After determining the reaction of the extract, quantities of 100th normal lactic acid or bicarbonate of soda were added to 10 c.c. portions of the extract, and sterile water to bring the volume up to 11 c.c. One c.c. of a suspension of *Bac. prodigiosus* was added, and this brought the volume up to 12 c.c., upon which the quantities of acid and alkali, which were added, had been based. The bottles were incubated at 22° for 20 hours, when counts were made, and these were calculated in terms of the neutral extract.

EXPERIMENT XX.

Reaction of extract (Fuller's scale).	Growth of *Bac. prodigiosus*, 20 hours at 22°.			
	a	b	c	average.
−0·3	56	43	53	50
−0·2	50	67	60	60
−0·1	93	71	70	78
0·0	100	100	100	100
+0·1	86	88	77	83
+0·2	33	44	21	33
+0·3	3	10	3	5

The numbers in the three tests are not uniform, but they serve to show the probable variation that the effect of dilution has upon an acid or alkaline extract. For example, in the ten days' test with *Penic. cladosp.* (xix.), the numbers with 80% were 187, and with 20%, 38. The reaction-curve passes through 86 for +0·1, 91 for +0·08, and 99 for +0·02. A slight calculation shows that the numbers at the 80% dilution are higher by 6%, and at the 20% dilution higher by 15% than they should be on account of the reduction of the acidity by the mere dilution. In these weakly acid extracts, however, small differences such as these are negligeable, as the counts themselves are liable to greater fluctuations.

A stage has been reached in the investigation at which it is made clear that bacteria and moulds do not produce toxins, or, if they do, the toxin is not capable of being demonstrated either in nutrient solutions or in vegetable-humus. Any resemblance to toxicity is probably caused by an alteration in the reaction of the medium, and, to such alterations of reaction, the test-organism is very sensitive.

THE GROWTH OF AMŒBÆ.

The attempt to obtain toxic substances among the byproducts of certain bacteria and moulds had not been so successful as had been wished; in fact, it had been decidedly unsatisfactory. Either the methods of producing the desired bodies were at fault, or the micro-organisms were not such as would give the desired results, although they had been selected as being the most likely to do so. It is known that bacteria can give out bacteriolytic

substances, as for example, *Bac. pyocyaneus*, and moulds may be capable of doing the same. But these are not the only inhabitants of the soil. There are certain protozoa, and with the failure to obtain a decided and undoubted production from the bacteria and moulds, the attention was turned to the soil-fauna.

With regard to the possible activity of the members of the soil-fauna in this direction, we are faced with the fact, that the introduction of certain species of protozoa, *e.g.*, the Amœbæ and Colpodæ into sterile soils, does not bring about the condition that holds before sterilisation. The bacterial numbers, which become greatly increased as a result of the sterilisation, do not become reduced to the previous level when the protozoa are present. It is true that Goodey* has lately shown that the inhibiting factor, which determines the reduction of the bacterial numbers, becomes evident when the numbers of amœbæ approach and exceed 50,000 per gram of dry soil, but, at the same time, he says that the sterilised or disinfected soils are not suitable media for the production of the factor. The treatment of the soil with heat or with the volatile disinfectants so alters the soil, that it is not immediately suitable for developing the inhibiting factor, whatever it may be.

I had considered the possibility of soil-amœbæ being able to produce substances akin to the immune bodies of the animal pathologist, but, as experimental work failed to reveal any sign of toxic substances, and such immune bodies must be included in this category, I simply record the fact that such a possibility had been entertained, and that experiments with, and without, "vaccines" had been negative.

Some difficulty was met with in obtaining a "pure" culture of soil-amœbæ. Upon a former occasion, they had been cultivated in quantity in 1% hay-infusion, without any trouble, but, upon repeating the procedure, it was found that the medium was not well adapted for the purpose. The single cells generally disappeared overnight. The trouble was traced to the medium being too nutritive, thus permitting the accompanying bacteria to become too numerous and render the medium too alkaline. A

* Proc. Roy. Soc., 89 (B.616), p.297.

hay-infusion, in which the bacteria had grown for some time, had changed from having a faint original acidity of +0·25 to an alkalinity of −2·4. It was naturally assumed that the alkalinity developed overnight had been the cause of the disappearance of the amœbæ, but, when either saccharose, lactose, lactic acid, humic acid, or sodium phosphate was added, the trouble still remained, although it was not so pronounced in the presence of humic acid. The addition of potassium nitrate, mono- or di-hydrogen phosphate, or ammonium phosphate to make a 0·05% solution with the hay-infusion, also resulted in the disappearance of the amœbæ. It seemed at one time as if the smallest droplets gave the most satisfactory growths, that one amœba in a small droplet had a better chance of growing than one cell in a large droplet, and it was concluded that the hay-infusion was too strong. It is a recognised rule, in growing amœbæ, that a poor medium should be used in order to prevent the protozoön being overwhelmed with bacteria, but 1% hay-infusion is by no means considered to be a rich medium, especially when made from a rather poor sample of couch-grass. However, experiments showed that 0·2% hay-infusion was well adapted for growing amœbæ in mass-culture, although it produced rather delicate forms in single-cell work. One of my most successful starter-cultures was obtained by gradually adding 1% hay-infusion so that the bacteria were kept under as much as possible. An equally good starter was obtained by growing the cells in a 2% infusion of exhausted leaf-mould, the same as was used in some experiments about to be recorded.

During this part of the investigation, the effect of adding sodium chloride to the hay-infusion was tested. In one instance, the addition of 0·2% was beneficial, while a larger quantity destroyed the amœbæ. In another instance, the addition was injurious. A good culture-fluid was found in tap-water containing 0·05% asparagin with 0·11% K_2HPO_4. Although two of the original five cells died overnight, the remaining three cells increased to 39 in another day. In view of this, an experiment was made in which the asparagin was replaced by chloride, sulphate or nitrate of ammonia, nitrate of potash and urea. A

number of amœbæ were added to each droplet, and a trace of chalk. All the cultures did well, and, in course of time, the mobile forms encysted. The cells of the urea-test were vegetating long after the others, and the amœbæ in the ammonium chloride test also persisted longer, and finally disappeared without forming cysts.

A preliminary experiment was made with an extract of a partially exhausted leaf-mould, using a growth of amœbæ derived from a single cell. Although the solutions were tested from time to time in the customary manner for the influence of boiling and dilution, no definite information was obtained, and it was concluded that the culture-solution was too poor in nutrients to show or develop any signs of toxicity.

Vegetable-mould neutralised with lime (p.168) was then used. Each test contained the equivalent of ten grams of dry organic matter with an amount of water sufficient to cause the soil to adhere loosely to the sides of the containing bottle. This meant 73% of moisture. It was apparent from the results that the mould became too acid for the continued growth of the amœbæ. Forty thousand were added to each bottle, and, in five days, they had increased to 1·6 millions, in twelve days they had decreased to 136,000 mobile forms, and, by the twentieth day, they all had encysted. While this was going on, the extract, originally neutral, became more and more acid, doubtless due to the carbon dioxide, produced by the activity of the bacteria introduced with the amœbæ, reacting with the calcium humate, and liberating free humic acid.

The test was repeated with similar results. On the sixth day, the amœbæ had increased forty-four fold, and the reaction of the extract was $-0{\cdot}04$; on the thirteenth day, the amœbæ had encysted, and the reaction was $+0{\cdot}1$. The bacterial numbers gave no information.

Better results were obtained with an infusion of hay made by diluting a 1% infusion with tap-water to make a 0·2% solution. Tests were made on the sixth, twentieth, and forty-second days, but, as the two latter gave somewhat normal dilution-curves, they are not recorded.

Experiment xxi.

Soil-protozoön.	*Amœba limax.*	Numbers corrected for alkalinity.
Extract, boiled ...	575	575
Extract, raw	100	100
Extract, raw, 80% ...	387	277
Extract, raw, 50% ...	962	489
Extract, raw, 20% ...	3,252	1,205
Water-control ...	5,522	1,821
Bacteria added at start	712	204
Extract/water ratio ...	0·018	—
Reaction of extract ...	−0·38	−0·0

The figures are instructive, inasmuch as they show a considerable reduction in the number of bacteria originally added. There was something in the solution which was strongly toxic towards the test-organism added to the extract. It is unfortunate that the extract was so alkaline, for this undoubtedly clouds the issue, but, even when an allowance is made for it, according to the information previously obtained with extract of vegetable mould (p. 172), a strongly rising dilution-curve is still apparent.

The alkalinity was determined by boiling 25 c.c. of the extract with 35 c.c. of distilled water and 1 c.c. of centinormal sulphuric acid for ten minutes, rapidly cooling the solution, adding 1 c.c. of centinormal soda, and titrating back until the tint became that of the control. Phenolphthalein was used as the indicator. It gave a true indication of the reaction in extracts of leaf-mould, but, as will be shown subsequently, it is not so good for solutions of hay-infusion.

The experiments so far showed that some means must be adopted to eliminate the excessive alkalinity of the fermented cultures. On a previous occasion, humic acid had been used, but, as it had been soluble, it had not served the desired purpose. On standing, however, a solution of the acid had precipitated, and this precipitate of insoluble humic acid was washed and used. The employment of soil for maintaining a neutral reaction was suggested by the fact that, when it is put into hay-infusion and incubated, there is developed a mixed flora and fauna, the appearance of which is so healthy, that a pronounced acidity or alkalinity of the infusion is unlikely.

In one test, a small amount of sodium phosphate was added to the hay-infusion, but, from the appearance of the amœbæ, the salt seemed to have enhanced the alkaline effect, at any rate it favoured bacterial growth and rapidly destroyed the amœbæ.

The neutralisation of the alkalinity with lactic acid proved to be useless, for, in three days, the culture was as alkaline as before the addition.

The advantage to be gained by using insoluble humic acid or soil was tested with solutions obtained by diluting a 1% infusion of hay with nine volumes of water, that is they contained one part of hay per thousand.

Experiment xxii.

Protozoön	*Amœba limax.*							
Medium	Hay-infusion with humic acid.			Hay-infusion with soil.				
Test	1	2	3	4	5	6	7	8
Duration of test in days	10	14	24	8	12	15	22	36
Extract, boiled ...	635	503	869	955	1,749	2,160	637	3,477
Extract, raw ...	100	100	100	100	100	100	100	100
Extract, raw, 80% ...	107	115	108	117	151	106	87	122
Extract, raw, 50% ...	104	182	117	160	169	313	109	190
Extract, raw, 20% ...	89	203	129	264	451	665	156	494
Water-control ...	55	86	137	302	398	1,036	288	332
Bacteria added at start	9·6	40	20	52	188	99	42	35
Extract/water ratio ...	1·8	1·1	0·09	0·33	0·25	0·1	0·34	0·3
Reaction of extract ...	+0·0	+0·02	−0·04	+0·02	−0·02	−0·02	−0·05	+0·03

The tests were started with 500 c.c. of 0·1% hay-infusion, either a small quantity of washed humic acid (about 0·03 gram) or 25 grams of sterile soil and 10 c.c. of an amœba-culture. The latter represented from 30,000 to 50,000 mobile forms as estimated by the counts of later starter-cultures. The amœbæ in the earlier periods were not counted, but they grew well, and a count made on the sixteenth day showed 10,000 per c.c. in the humic acid, and 5,600 in the soil test. On the twenty-fourth day, the flasks contained 6,600 and 3,600 per c.c. respectively.

The results show a low reaction, and it was assumed that the fluids were approximately neutral. The humic acid tests do not

exhibit any pronounced degree of toxicity as judged by the effect of boiling, or dilution, or by the relation of the raw extract to the water-control. They are of the indefinite type which leads one to further experimenting in the hope of obtaining something more definite.

In the soil-tests, we have a direct evidence of toxicity in the twelve days' culture. The filtered extract was directly toxic, reducing the number of bacteria added at the start from 188 to 100. The effect of boiling the extract for an hour under a condenser, and of diluting the raw extract is also well shown in the increased nutritive effect. The toxic nature of the twelve days' culture is confirmed by that of the fifteen days', for, although the added bacteria are not decreased, they did not increase.

A trial was made to see if humus, such as vegetable-mould, would be useful for maintaining an approximate neutrality. Four grams of lime-treated mould were tried against 50 grams of sterile soil. In fourteen days, the humus test showed a reaction of $-0{\cdot}4$, and the soil $-0{\cdot}03$. Humus was therefore useless for the purpose. There was no evidence of toxicity in the extracts.

While dilute hay-infusion has been found to be very good for growing the amœbæ, and for obtaining evidence of toxin-formation, it seemed advisable to see if a simple nutrient would be as good. The preliminary tests in the cultivation of the amœbæ had shown that they grew well in urea and ammonium chloride and, with these, calcium nitrate was included as a nutrient, which would probably not produce an alteration in the reaction of the culture-medium. Solutions of urea, $0{\cdot}02\%$, ammonium chloride, $0{\cdot}036\%$, and calcium nitrate, $0{\cdot}056\%$ in tap-water, were prepared. These contained equivalent quantities of nitrogen. To 500 c.c. of these solutions, 50 grams of soil and 25 c.c. of an amœba-culture containing 75,000 mobile forms were added. Unfortunately the amœbæ did not increase in numbers and were rarely more than 330 per c.c. Extracts were prepared on the seventeenth day when all hope of their increase had been given up. The extracts had the following reactions, urea, $-1{\cdot}36$, ammonium chloride, $+0{\cdot}24$, and calcium nitrate, $+0{\cdot}04$. There

was no evidence of toxicity other than could be accounted for by the reaction. The experiment did not indicate that any of these chemicals would be of any value in the research.

The Influence of Aeration.

With the idea of determining the influence of aëration upon the production of toxin, a quantity of dilute (0·2%) hay-infusion was infected with a culture of amœbæ, 50 grams of soil were added, and the bottle containing the test was attached to an aspirator, which caused a few bubbles of air to pass through the liquid every few minutes. A control-bottle was allowed to stand in the laboratory. They were ordinary litre-bottles, and the 500 c.c. of infusion was $2\frac{3}{4}$ inches deep. Portions of the fermented liquids were filtered through porcelain on the fifth day, and again on the eighth day, with the following results.

Experiment xxiii.

Treatment	Aërated.		Not aërated.	
Duration of test in days ...	5	8	5	8
Extract, boiled	33	122	1,690	9,550
Extract, raw	100	100	100	100
Extract, raw, 80% ...	113	126	102	137
Extract, raw, 50% ...	117	144	160	429
Extract, raw, 20% ...	108	356	229	600
Water-control	74	242	152	574
Added at start	17	21	38	49
Extract/water ratio ...	1·3	0·4	0·6	0.2
Reaction	+0·2	+0·06	−0·06	−0·02

The solutions were twice the strength of those of Experiment xxii., but whether this accounted for a slow growth of amœbæ in the non-aërated test or not, cannot be said. None were seen on the fifth day, 330 on the eighth, and 2,300 per c.c. on the twelfth. In the aërated test, the amœbæ grew well; 3,000 were noted on the fifth, and 9,300 per c.c. on the seventh day. Aëration induced an acid, and its absence an alkaline reaction. The acidity was so high on the fifth day that, on the sixth, the aëration was stopped. This appeared to have had an effect upon the dilution-curve.

The greater growth of amœbæ and the smaller probability of the production of a toxic effect in aërated cultures raised the idea that, like yeast-cells, the reproductive may be inversely proportional to the physiological activity. So another experiment was started, and, as a variation, a flask was included which was infected with a culture of bacteria only, derived from a protozoön-free droplet of a starter. The extracts were tested on the sixth day.

EXPERIMENT XXIV.

Micro-organism ...	*Amœba limax.*		Bacteria only.
Method	Aërated.	Not aërated.	Not aërated.
Extract, boiled ...	1,168	2,039	3,453
Extract, raw	100	100	100
Extract, raw, 80% ...	75	109	106
Extract, raw, 50% ...	99	123	87
Extract, raw, 20% ...	103	199	260
Water-control	87	100	78
Added at start... ...	15	17·5	13·7
Extract/water ratio ...	1·15	1·0	1·27
Reaction	+0·04	−0·05	+0

The numbers bear out the contention that, if toxicity is to be obtained, it will not be as the result of aëration. The reaction went on as in the previous case, aëration producing acidity, and no aëration, alkalinity in hay-infusion. The examination of the culture-fluids showed that the amœbæ had begun to encyst in the aërated, and that 1,000 mobile forms per c.c. were in the other.

A further test was made upon the same lines; as a variation, a deep layer of fluid was used without aëration in order to accentuate the conditions. The method at this time had been to use 700 c.c. of fluid contained in a bottle of about 1,200 c.c. capacity, and, in this, the fluid had a depth of 9 cm. In the deep test, 1,700 c.c. were used, and in an ordinary winchester this had a depth of 16 cm. In the latter, the amœbæ grew slowly, the first indication being obtained on the thirteenth day, when 330 per c.c. were noted. On the seventeenth, they had risen to 1,000. The aërated test showed 5,300 on the fourth, and, without aëration, the first evidence, 330 per c.c., was obtained on the eleventh day.

EXPERIMENT XXV.

	No amœbæ.		*Amœba limax.*							
	No aëration.		No aëration.		Aërated.		No aëration, deep layer.			
Period of incubation in days	4	7	4	7	4	7	6	9	13	26
Extract, boiled	757	837	518	1,883	420	64	6,193	863	1,286	734
,, raw	100	100	100	100	100	100	100	100	100	100
,, ,, 80%... ...	86	84	95	91	92	53	261	131	105	120
,, ,, 50%... ...	168	81	147	221	95	193	145	163	157	177
,, ,, 20%... ...	579	141	559	224	157	227	177	131	162	302
Water-control	210	118	125	261	175	86	137	175	71	215
Added at start	83	18	49	41	69	13	54	27	15	28
Extract/water ratio ...	0·47	0·85	0·80	0·38	0·57	1·15	0·73	0·57	1·40	0·46
Reaction...	−0·02	−0·3	−0·04	−0·3	−0·04	+0·04	−0·04	−0·3	−0·34	−0·26

Taking the figures as a whole, there is not sufficient difference between them to justify the consideration that any toxin had been produced by the amœbæ, for rises in the dilution-curve are obtained in their absence. In view of later experience, the rises might well have been caused by the reaction of the culture-fluid.

About this time, it became evident that the method of determining the reaction by the use of phenolphthalein, as the indicator, might be faulty when hay-infusion was used, and that results based upon the reaction of a medium such as extract of vegetable-mould did not hold for another medium, such as hay-infusion. The method had been to add a few drops of phenolphthalein to a portion of the extract, and boil for ten minutes. If the solution became purple, another portion was boiled with acid, and titrated back. If it only became slightly reddened or tinted, it was titrated without boiling with acid. When methyl-orange was employed as the indicator, the reaction-numbers were much higher.

It became necessary to examine the influence of various strengths of hay-infusion, as a direct toxic action had been found in a 0·1% solution, and had not again been obtained in 0·2% solutions. The bottles received 700 c.c. of infusion, 60 grams of sterile soil, and 20 cc. of a starter containing 112,000 mobile amœbæ.

EXPERIMENT XXVI.

Strength of infusion ...	0·05%.		0·1%.		0·2%.		
Duration of test ...	5	8	7	10	7	10	17
Extract, boiled ...	610	135	729	353	1,016	2,038	1,028
,, raw	100	100	100	100	100	100	100
,, ,, 80% ...	—	111	94	219	98	425	111
,, ,, 50% ...	377	118	174	216	143	586	167
,, ,, 20% ...	150	69	298	28	343	136	250
Water-control ...	122	61	219	86	400	154	181
Added at start ...	16	8	28	11	51	20	28
React'n, phenolphthalein	- 0·02	- 0·03	- 0·02	- 0·02	- 0·02	- 0·05	- 0·06
,, methyl-orange ..	- 0·52	- 0·23	- 0·32	- 0·30	- 0·52	- 0·39	- 0·52
Extract/water ratio ...	0·8	1·6	0·5	1·1	0·2	0·6	0·5
Amœbæ per c.c. ...	500	1,000	1,830	2,000	660	5,600	2,000

There was no direct toxic effect in any of the tests, and, so far as the possibility of obtaining such by using different strengths of infusion are concerned, the strongest appears the most likely. The ten-days' extract of the 0·2% solution gave more favourable numbers than any of the others. In this, the amœbæ were the most numerous.

A further test with 0·1% hay infusion was made, but in it the amœbæ did not grow quite so well as on the former occasion when the direct toxic action was obtained. The counts showed 830 per c.c. on the seventh, and 1,000 on the eighth day. No evidences of toxicity were obtained on the eighth or twenty-first days, and it must be concluded, that a definite toxicity cannot be demonstrated by growing bacteria or amœbæ in the usual culture-fluids.

The Influence of Reaction.

Much has yet to be found out regarding the influence of reaction upon bacterial growth, and the reason for the rise in the dilution-curves of the extracts. As the infusions and culture-solutions are generally made with tap-water to supply a small quantity of saline matter accepted as being necessary for the growth of micro-organisms, a beginning was made with it. Tap-water is known to be alkaline, but the extent of the influence of its faint alkalinity is not known. The nutrients were increased by preparing infusions of hay, and these were made sufficiently weak to enable useful counts to be obtained. A 1% infusion of hay was diluted with 99 parts of porcelain-filtered, distilled and tap-water, thus obviating heat-effects. Portions were treated with lactic acid and with ammonia so as to bring up the final volume to a definite reaction, and sown with a definite number of cells of *Bac. prodigiosus*. They were incubated at 22° for 20 hours, and counted. In tabulating the results, the highest counts were taken as 100.

EXPERIMENT XXVII.

Reaction.	Distilled water.	Tap-water.
+0·3	11	16
+0·2	18	29
+0·1	44	100
+0·05	59	—
0·0	100	5
−0·05	44	—
−0·1	28	0·1
−0·2	7	0·05
−0·3	0·6	0·02
Reaction of the untreated diluted infusion to methyl-orange	±0·0	−0·20
To phenolphthalein, direct	—	−0·03
To phenolphthalein, indirect	—	−0·14

The distilled water test shows that the bacteria grow best in a neutral solution, and the tap-water test that neutrality is obtained when approximately +0·1° of acid has been added. The curves of these numbers are steep on both sides of the approximately neutral line, indicating that a slight difference in the reaction of an extract will make a great difference in the growth-numbers. As it is impossible to obtain a neutral reaction in the cultivated extracts, it would be necessary to neutralise them before dilution in such tests as have been made. It is not clear how this could be done, for, in the solution under examination, the bacteria showed 0·1° of alkalinity, methyl-orange showed 0·2°, phenolphthalein by direct titration after boiling, 0·03°, and by indirect or back titration, 0·14°.

The experiment would be incomplete without the inclusion of others showing the influence of diluting distilled and tap-water infusions with distilled water, as is customary.

EXPERIMENT XXVIII.

	Distilled water.		Tap-water.	
			Raw.	Boiled.
	a	b	a	b
Solution, boiled	26	—	1,020	131
,, unboiled	100	100	100	100
,, ,, 80%	75	86	140	234
,, ,, 50%	—	42	1,160	207
,, ,, 20%	27	18	2,880	182
Distilled water-control	2	3·5	263	32
Added at start	0·4	2	50	20
Solution/water-ratio	47	28	0·4	2·5
Reaction to methyl-orange ..	- 0	- 0	- 0·16	- 0·24
Reaction to phenolphthalein, direct	- 0	- 0	- 0·05	- 0·11
Reaction to phenolphthalein, indirec	- 0	- 0	- 0·16	- 0·24

The boiled tap-water infusion, "b," was boiled three times upon successive days, as is usual in preparing such culture-media. Tests "a" were made eleven days before tests "b." By an accident, the "b" tests were incubated at 26° instead of 22° as in "a," and this should be borne in mind when considering the increase of the unboiled numbers over the start.

The distilled-water numbers are considered to be normal, for they show a gradual fall as the nutrients are weakened by dilution with water. The tap-water curves rise as the alkalinity is weakened. The numbers of the raw tap-water test indicate that, after all due allowances are made, it is of a toxic nature, which is probably not entirely traceable to the alkalinity. The numbers of the boiled-water test are not so pronounced, and are much the same as have been obtained in previous experiments with bacterial and protozoön cultures. It should, however, not be forgotten that many of these gave normal curves.

A set of experiments were started when it had become evident that, in all probability, the reaction had more to do with the symptoms of toxic effect than anything else. In the endeavour to get round any individual action of the bacteria, flasks of dilute (0·1%) hay-infusion, made with distilled water to avoid the action of tap-water, were seeded with an amœbæ-culture, and with a bacterial culture derived originally from a protozoa-free

droplet of soil-suspension. The latter were thus controls. The amœbæ-cultures were twice seeded with amœbæ, once, at the start, and again on the second day, as the first seeding did not seem to have been successful. They grew slowly at 18°, and exhibited a twenty-fold increase on the tenth day, and a fifteen and ten-fold increase on the thirteenth day with the humic acid and humic acid + soil respectively. They were tested on the fourteenth day.

EXPERIMENT XXIX.

	Humic acid.		Humic acid + soil.	
	Amœbæ.	No amœbæ.	Amœbæ.	No amœbæ.
Extract, raw	100	100	100	100
,, ,, 50%	192	125	184	183
Water-control	133	82	98	88
Added at start	16	10	11	10
Extract neutralised with lactic acid	104	127	198	243
Extract/water-ratio	0·75	1·2	1·0	1·1
Reaction to methyl-orange ...	−0·15	−0·28	−0·21	−0·28

The addition of soil to the culture-fluids had no influence, one way or the other, in increasing or decreasing the numbers, and its use appears to be of no value. The experiment shows pretty clearly that any toxic effect is not caused by the amœbæ, but rather by the bacteria which always accompany the protozoa. It is doubtful if the alkalinity, as indicated by methyl-orange, is a true index, but, if accepted as true, the neutralised solutions were certainly more nutritive than the unneutralised, for the numbers were higher. With a better indicator, a lower reaction might have been obtained and higher numbers furnished in the neutralised tests. At any rate, it is pretty safe to conclude, that the rise in numbers upon dilution is largely, if not entirely, due to the lessening of the alkalinity of the filtered extracts.

REACTION-EXPERIMENTS.

The reaction of the soil-extract is never constant, but varies from day to day, probably within certain limits. For example, an extract was made on November 30th, 1917, by taking 300

grams of garden-soil and 300 c.c. of distilled water. The two were shaken 300 times during an hour, and filtered. The reaction to methyl-orange was −0·14, although a paste of the soil was acid to litmus.

A similar extract, made on December 6th, had a reaction to methyl-orange of −0·24. This extract was examined, with the following results.

EXPERIMENT XXX.

Soil-extract, boiled	21
,, raw	100
,, ,, 80%	100
,, ,, 50%	64
,, ,, 20%	12
Water-control	0·5
Added at start	0·7
Extract/water ratio	206

Quantities of lactic acid and of carbonate of soda were added to vary the reaction, and the treated extracts were seeded with the test-organism in the usual manner. The following numbers were obtained after the usual 20 hours' incubation at 22°.

EXPERIMENT XXXI.

Acid or alkali added.	Net reaction.	Bacterial growth.
+0·4	+0·16	1·5
+0·3	+0·06	6·3
+0·2	−0·04	17
+0·1	−0·14	49
+0·05	−0·19	100
0·0	−0·24	81
−0·05	−0·29	31
−0·1	−0·34	15
−0·2	−0·44	1·3

The true neutral point was reached by adding 0·06° of acid, that is to say, the methyl-orange indication was −0·19 in excess.

The effect of shaking up various quantities of soil and water was tested, to see the differences in the reaction of the extracts. Round numbers were taken, but, as the solid contained 8% of moisture, a correction was made.

Proportion of soil to water, round numbers ...	2 : 1	1 : 1	1 : 2
Proportion of soil to water, calculated for dry soil...	1 : 0·63	1 : 1·17	1 : 2·26
Reaction to methyl-orange	−0·24	−0·18	−0·12
Reaction to phenolphthalein, direct ...	−0·044	−0·016	−0·008

Curves of these numbers show that the reaction of the soil-water, equivalent to a ratio of 1 : 0·087 for the dry soil to 8% of moisture, would be between − 0·4 and − 0·5 for methyl-orange, and about − 0·09 for phenolphthalein; yet the soil was acid to litmus.

The reaction to phenolphthalein was examined somewhat closely, after the suspicion was raised that the reaction of the solution had more to do with the phenomenon of toxic action than had been supposed to exist. It was found to be untrustworthy, as much depended upon the rate at which the solutions were boiled, previously to cooling and titration. For example, covered beakers containing 50 c.c. of tap-water were boiled slowly and rapidly, cooled and titrated.

Duration in minutes ...	10	20	30
Slow boiling	-0·022	-0·048	-0·071
Rapid boiling	-0·076	-0·105	-0·118

Much, therefore, depends upon the method and time of boiling in expelling the carbon dioxide. In the tests previously recorded, the extracts were boiled in an open beaker for ten minutes at a speed intermediate between slow and rapid boiling. They probably do not indicate the true alkalinity, but, for that part, it has been shown that the same would have occurred by using methyl-orange.

A twenty-one-days' culture of bacteria and amœbæ was filtered, and the extract treated with lactic acid in decreasing quantities, seeded with the test-organism, incubated and counted. It had a reaction to methyl-orange of − 0·16°, and to phenolphthalein, direct, of − 0·04°.

Experiment xxxii.

Lactic acid added.	Net reaction.	Bacterial counts.
0·31	+0·15	8
0·26	+0·10	19
0·21	+0·05	41
0·16	0·0	46
0·11	−0·05	40
0·06	−0·10	100
0·0	−0·16	53

The experiment shows that, in the case of a fermented culture-fluid, the lessening of the alkalinity by 0·06° produced neutrality. The methyl-orange indication was −0·10° too high. A high indication was also obtained with soil-extract in Experiment xxxi., and with tap-water in Experiment xxvii.

The acidity of the extracts of sterilised vegetable-moulds (Experiments xv., xvi.) led to an examination being made of the extracts of soil which had been sterilised in the same manner, viz., for two hours at 130°.

Two hundred gram-portions were put into sterile bottles with 10 c.c. of a suspension of *Amœba limax*, containing 10,660 mobile forms per c.c., and 40 c.c. of water. The soil thus had an excessive amount of moisture, 20%; it usually contains from 8 to 10%. The bottles were kept at laboratory-temperature, which varied from 18-23°.

Extracts were made in the usual manner by adding 200 c.c. of distilled water, shaking frequently during an hour, and filtering through porcelain. The extracts were seeded with *Bac. prodigiosus*, incubated and counted.

Experiment xxxiii.

Duration of test in days	3	53
Extract, raw	100	100
Extract, raw, 50%	240	48
Water-control	1	1·2
Added at start	0·16	0·14
Extract/water ratio	110	153
Reaction to methyl-orange ...	+0·3	−0·8

The point to be noted is the change in the reaction of the extracts. It changed from being comparatively strongly acid on the third day, to strongly alkaline on the fifty-third. When the extract of the latter was treated with lactic acid to neutralise the apparent alkalinity, the number obtained was 0·07 as against 100 for the non-neutralised extract; that is, for every seven bacteria in the neutralised, there were 14,000 in the alkaline extract. The acid had clearly been added in excess, the $-0{\cdot}8^{\circ}$ being far from a true indication.

This confirms the result obtained in another place, that the reaction of a soil-extract, as judged by methyl-orange, is not the true reaction. Mobile amœbæ were seen in the soil on the fifty-third day.

Conclusion.—It has been shown that certain soil-bacteria, moulds, and amœbæ, all reasonably supposed to be capable of furnishing substances of a toxic nature, were grown in various media and under varying conditions; and, in all cases, the signs of toxicity which became manifest could be attributed to an alteration in the reaction of the media.

The test-organism, *Bac. prodigiosus*, grows best in a neutral medium, and an indicator is required which will indicate strict neutrality. The methyl-orange numbers are too high, and the phenolphthalein too low. Small divergences from the neutral point strongly affect the growth.

The humus of leaf-mould contains two types of humic acid; one absorbs alkali from alkaline carbonates, and the other from alkaline carbonates and hydrates. These were present to the extent of one part of the former to three of the latter. Heating the humus increases the amount of acid, and the increase is largely soluble in water.

The effect of reaction is quite of a different order from the evidence of toxic action obtained in former researches.

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